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Clinical Implications of Coinfection With a Novel DNA Virus (TTV) in Hepatitis C Virus Carriers on Maintenance Hemodialysis

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A novel hepatitis-associated DNA virus, designated as transfusion-transmitted virus (TTV), was identified recently. We investigated the frequency of TTV viremia in hepatitis C virus (HCV) carriers on maintenance hemodialysis to determine whether TTV coinfection has any clinical relevance. The subjects were 50 hemodialysis patients who had been followed over 4 years after diagnosis of HCV infection. Stored serum samples derived from each patient every 12th month after enrollment were subjected to polymerase chain reaction to amplify TTV DNA and HCV RNA. At enrollment, TTV viremia was detected in 24 (48%) HCV-positive patients irrespective of the number of previous blood transfusions and the duration of hemodialysis. The presence of TTV viremia had no relation to serum alanine aminotransferase (ALT) levels, HCV viremic levels or HCV genotypes. After enrollment, HCV infection persisted in all patients over the 4-year follow-up period, whereas spontaneous resolution of TTV infection was observed in 7 (29%) of the 24 TTV viremic cases (annual rate 7.3%, 95% confidence interval [CI] 0.8–25.5%). Evidence for TTV infection was found in 4 (15%) of the 26 TTV nonviremic patients (annual incidence 3.9%, 95% CI 0.1–19.6%). The relationship between the ALT profile and TTV infection during follow up was not evident. Active TTV coinfection occurs frequently in HCV carriers undergoing hemodialysis but exerts no biochemical or virological influence on the underlying hepatitis C. Lack of disease association and the frequent spontaneous resolution of infection suggest that the clinical significance of TTV infection remains unclear. ***J. Med. Virol. 59:431–436, 1999.***

KEY WORDS: viral hepatitis; hepatitis C; non-A-G hepatitis

INTRODUCTION

In December 1997, a novel hepatitis-associated parvovirus-like agent was identified by investigators in Japan and provisionally designated as transfusion-transmitted virus (TTV) [Nishizawa et al., 1997]. TTV is an unenveloped, single-stranded DNA virus with at least 3,700 nucleotides. Hitherto, the presence of TTV DNA in serum has been examined by polymerase chain reaction (PCR), and the global distribution of TTV has been revealed [Charlton et al., 1998; Naoumov et al., 1998; Simmonds et al., 1998]. Although TTV was isolated from serum samples from a patient (TT) with post-transfusion hepatitis of unknown etiology and the viremia coincided with modest increases in serum alanine aminotransferase (ALT) levels [Nishizawa et al., 1997], the association of TTV with disease remains to be clarified. The parenteral route of TTV transmission has been reported, and TTV DNA prevalence seems to be high in populations at risk of parenteral exposure to infectious agents [Okamoto et al., 1998b; Simmonds et al., 1998]. However, the exact mode of transmission and the natural course after infection remain unresolved. To address these issues, we monitored TTV infection over 4 years in hepatitis C virus (HCV) carriers on maintenance hemodialysis.

MATERIALS AND METHODS

Subjects

The subjects were 50 HCV carriers on chronic outpatient hemodialysis, who had been enrolled consecutively and undergone detailed follow-up over 4 years at Inoue Hospital, Osaka, Japan. TTV infection in these patients was studied using stored serum samples. At

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enrollment, they were definitely diagnosed as having HCV infection (HCV antibody positive on second-generation assay [Ortho Diagnostic Systems Co., Ltd., Tokyo, Japan] and serum HCV RNA positive by PCR), were not infected with hepatitis B virus (HBV) (hepatitis B surface antigen [HBsAg] negative), had no history of administration of hepatotoxic drugs or alcohol abuse (>80 g/day), and showed no evidence of autoimmune liver disease. The causes of their renal failure were chronic glomerulonephritis ($n = 40$), diabetic nephropathy ($n = 5$), obstructive nephropathy ($n = 3$) and polycystic kidney disease ($n = 2$). After enrollment, all patients were monitored over 4 years and underwent physical examination and routine laboratory tests including serum ALT activity every second week. Serum HCV RNA and HBsAg were tested every third to sixth month. All patients were persistently infected with HCV but remained seronegative for HBV until the end of the follow up. In each case, there was no apparent nonviral cause of hepatocellular injury during the entire follow-up period. Serum samples obtained from each patient at enrollment and then every 12th month were stored at −80°C and subjected to TTV PCR.

Laboratory Methods

Biochemical and virological tests were performed on serum samples drawn immediately before hemodialysis. Serum ALT activity was measured at Biomedical Laboratories (Kawagoe, Japan) by ultraviolet absorption spectrophotometry. In the present study, the upper normal limit of ALT activity (45 U/L for healthy adults) was corrected for hypoaminotransferasemia in patients undergoing hemodialysis. Sera from 400 patients undergoing hemodialysis, who were negative for serum HCV RNA and HBsAg and free of nonviral causes of hepatocellular injury, were subjected to measurement of ALT activity. Thus, the upper normal limit was reset at 25 U/L corresponding to the mean + 2 SD for a normalized distribution of ALT activity in the population.

Serum HCV RNA sequences were detected by reverse-transcription PCR as described elsewhere [Hagiwara et al., 1993]. Primers were derived from the 5′-noncoding region of the published sequence [Takamizawa et al., 1991]: antisense primer 5′ATGGTGCACGGTCTACGAGACCTCC3′ and sense primer 5′CACTCCCCTGTGAGGAACTACTGTC3′. The PCR mixtures were amplified for 40 cycles (94°C for 30 sec; 55°C for 60 sec; 72°C for 60 sec), followed by a 10-min final extension at 72°C. A portion of the PCR products was fractionated by agarose gel electrophoresis, transferred onto a nylon membrane, hybridized to a ^{32}P-labeled HCV complementary DNA between the two primers, and autoradiographed. Quantification of serum HCV RNA was carried out using a branched DNA (bDNA) assay (Quantiplex HCV-RNA, Chiron Corporation, Emeryville, CA). Specimens with quantification values over the cut-off value (350,000 HCV RNA Eq/ml) were considered positive. The results were expressed as $\log_{10}$ (HCV RNA equivalent per milliliter). An arbitrary volume of 175,000 Eq/ml was attributed to the serum samples judged positive by reverse-transcription PCR but negative by bDNA assay. HCV RNA-positive serum samples were further subjected to a serum-based genotyping assay using genotype-specific NS4 antibodies (Immucheck-HCV Gr, International Reagent Corporation, Kobe, Japan).

Serum TTV DNA sequences were detected by PCR according to a method described previously [Okamoto et al., 1998b]. TTV-specific hemi-nested primers were derived from two regions conserved between the most divergent variants of TTV described to date. In brief, sense primer NG059 (5′ACAGACAGAGGAGAAGGCAACATG3′) and antisense primer NG063 (5′CTGGCATTTTACCATTTCCAAAGTT3′) were used for the first round. The amplification was for 35 cycles at 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec, followed by a 7-min final extension at 72°C. The second round was with another sense primer NG061 (5′GGCAACATGTTATGGATAGACTGG3′) and the same antisense primer NG063. A portion of the first round PCR was amplified for 25 cycles at 94°C for 30 sec, 60°C for 45 sec, and 72°C for 45 sec, with a 7-min final extension at 72°C. The size of the first-round PCR was 286 bp, and that of the second-round PCR was 271 bp. The amplicons were electrophoresed in 3% agarose gel, stained with ethidium biomide, and photographed under ultraviolet light. All assays for TTV were performed in a blind fashion.

Statistical Analysis

The chi-square test with Yates' continuity correction or Fisher's exact test (if an expected cell was less than 5) was used for analysis of discrete variables. The Wilcoxon nonparametric test was used to compare continuous variables. A value of $P < .05$ (two-tailed) was considered to indicate significance.

RESULTS

Of the 50 HCV carriers on maintenance hemodialysis, 24 (48%) patients tested positive for serum TTV DNA at enrollment. The 50 patients studied had undergone hemodialysis in three hemodialysis sections located on different floors. TTV infection was found in 10 (48%) of the 21 patients from section 1, 14 (58%) of the 24 patients from section 2, and none of the five patients from section 3. Table I shows the baseline patient characteristics in relation to the presence of TTV coinfection. No significant difference was seen between TTV-positive and -negative patients with respect to age, sex, and histories of blood transfusions. Many in each group had a history of blood transfusions (88% and 85%, respectively), and the number of previous blood transfusions was the same for the TTV-positive group (median 3, range 0–45) and TTV-negative group

TABLE I. Baseline Clinical and Virological Features of HCV-Infected Hemodialysis Patients in Relation to TTV Coinfection

Characteristics	TTV DNA (+) (n = 24)	TTV DNA (–) (n = 26)	*P*
Age (years)	55 (25–66)	55 (36–77)	NS
Sex (M/F)	12/12	11/15	NS
History of blood transfusion	21/24 (88%)	22/26 (85%)	NS
Incidence of blood transfusion	3 (0–45)	1 (0–49)	NS
Duration of haemodialysis (months)	138 (12–270)	136 (15–252)	NS
Serum ALT level (U/L)	21 (8–68)	22 (4–72)	NS
No. with normal ALT[a]	16/24 (67%)	17/26 (65%)	NS
HCV viremic level			
RT-PCR+/bDNA–	4 (17%)	6 (23%)	NS
bDNA+	20 (83%)	20 (77%)	
HCV RNA titers (Eq/ml)	$10^{6.0}$ (RT-PCR+/bDNA–to$10^{7.2}$)	$10^{6.4}$ (RT-PCR+/bDNA–to$10^{7.8}$)	NS
HCV genotype			
Genotype 1	20 (83%)	17 (65%)	
Genotype 2	2 (8%)	4 (15%)	NS
Unclassified	2 (8%)	5 (19%)	

Quantitative data expressed as median (range). HCV, hepatitis C virus; TTV, transfusion-transmitted virus; ALT, alanine aminotransferase; NS, not significant.
[a]The upper normal limit of ALT activity was reset at 25 U/L for patients on maintenance hemodialysis.

(median 1, range 0–49). On each occasion, two units of packed erythrocytes were transfused for the treatment of anemia associated with renal failure. The duration of hemodialysis ranged between 12 and 270 months (median 138) for the TTV-positive group and between 15 and 252 months (median 136) for the TTV-negative one with no significant difference. Serum ALT activity was the same for the TTV-positive and -negative groups was within the corrected normal range in 67% and 65% patients, respectively. There was no difference in HCV viremic levels and the HCV genotype prevalence between the two groups.

In the present study, HCV infection, as diagnosed by serum HCV RNA PCR, persisted over the 4-year follow-up period in all of the 50 patients enrolled. TTV infection was further monitored by PCR in the study population (Fig. 1). Seven of the 24 patients, who tested positive for serum TTV DNA at enrollment, cleared TTV DNA in the serum during follow up. The probability of TTV clearance was 29.2% (95% confidence interval [CI] 12.6–51.1%) at 4 years in contrast with 0% (95% CI 0–7.1%) for HCV clearance. As for the 26 patients negative for serum TTV DNA at enrollment, four became positive after enrollment and remained positive during follow up. The probability of developing TTV infection was 15.4% (95% CI 4.4–34.9%) at 4 years. Three of the four cases were from hemodialysis section 1, and the remaining one from section 3. There was no difference in the incidence of TTV infection among the hemodialysis sections. Thus, the follow-up study revealed that spontaneous resolution of TTV infection (annual rate 7.3%, 95% CI 0.8–25.5%) as well as development of active infection (annual incidence 3.9%, 95% CI 0.1–19.6%) occurred in the study population. The baseline clinical and HCV virological features listed in Table I had no relation to the occurrence of TTV clearance in infected patients and that of TTV infection in uninfected patients.

Figure 2 shows the clinical course of the 11 patients in whom TTV clearance or active infection occurred during follow up. Patients 1–7 cleared TTV at 1–4 years after enrollment. Intermittent serum ALT elevation persisted after TTV clearance in patients 1, 5, and 7. Patients 2, 3, 4, and 6 retained normal or near-normal ALT levels over the entire follow-up period. Patients 8–11 showed development of active TTV infection. No blood transfusion had been given during the follow-up period to patients 8–10, who became positive for serum TTV DNA at 2–3 years after enrollment. Patient 11 had received blood transfusions at 4 and 7 months before he first tested positive for serum TTV DNA at 4 years. Patients 8 and 9 had normal or near-normal ALT levels over the entire follow-up period. In patients 10 and 11, intermittent ALT elevation persisted over the follow-up period. Thus, neither TTV clearance nor development of active infection were associated with liver disease activity. Liver disease activity during follow up was further evaluated for the 17 patients with persistent TTV infection and the 22 patients who remained free of TTV infection. The mean ALT value over the 4-year follow-up period was the same for the persistently TTV-positive group (median 18, range 8–33 U/L) and TTV-negative group (median 19, range 7–48 U/L). Both groups showed similar patterns of ALT fluctuation. Three (18%) patients in the TTV-positive group and four (18%) in the TTV-negative group had persistently normal ALT levels, whereas four (24%) in the TTV-positive group and nine (41%) in the TTV-negative group showed fluctuating ALT levels of more than two times the upper normal limit.

DISCUSSION

The preliminary data suggested that TTV is transmitted mainly via a parenteral route [Okamoto et al., 1998b; Simmonds et al., 1998]. Patients on maintenance hemodialysis are at a high risk of infection with blood-borne hepatitis viruses. When TTV infection was studied in hemodialysis patients who were monitored

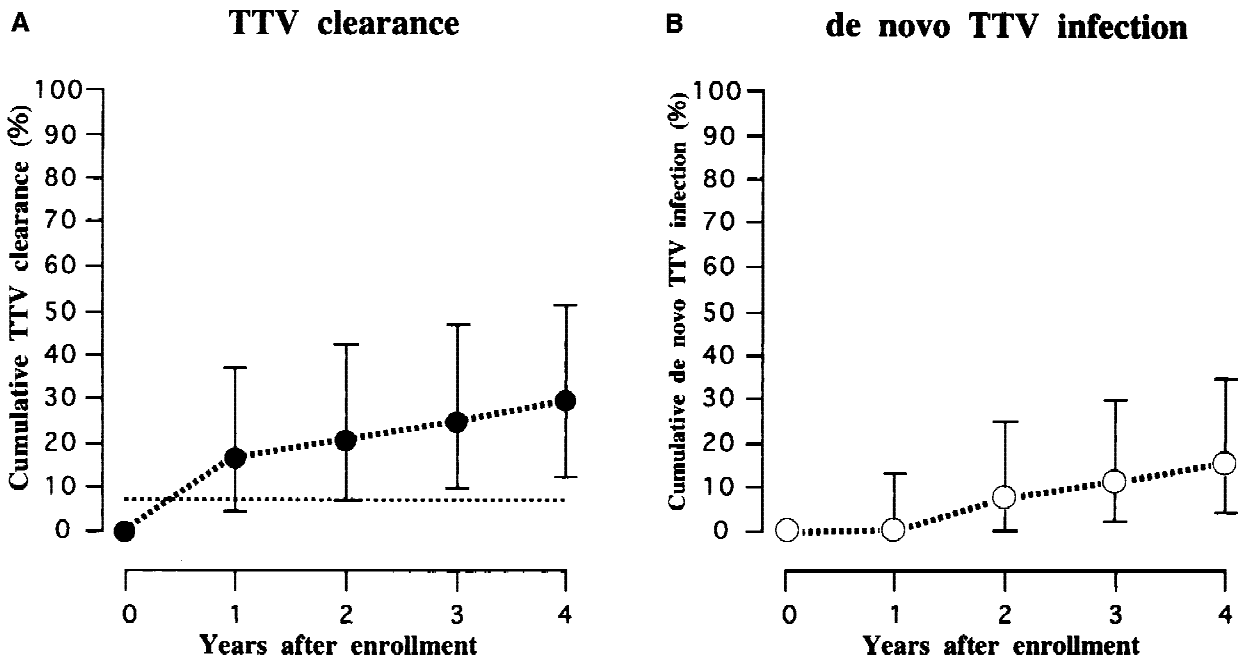

Fig. 1. Cumulative transfusion-transmitted virus (TTV) clearance (with 95% confidence intervals [CI]) in 24 patients infected with TTV at enrollment **(A)** and cumulative de novo TTV infection (with 95% CI) in 26 TTV-uninfected patients **(B).** The dashed horizontal line in (A) indicates the upper limit of 95% CI for hepatitis C virus clearance in the study.

for HCV infection, coinfection was found in 48% at enrollment. This prevalence is higher than the 12% for Japanese blood donors, which was initially reported using the same PCR method [Okamoto et al., 1998b]. The present study revealed further that TTV infection occurred in 15% (4/26) of uninfected patients at 4 years, and the annual incidence was estimated at approximately 3.9% (95% CI 0.1–19.6%). Although circumstantial evidence of TTV transmission via a parenteral route is strong, TTV infection was found irrespective of the incidence of previous blood transfusions and the duration of hemodialysis. Only one of the four patients who acquired TTV infection after enrollment had had blood transfusions that might have been involved in TTV transmission. Thus, risk factors for parenteral transmission were not associated with the occurrence of TTV infection in this study. TTV infection can be found in blood donors without previous blood transfusions. The excretion of TTV into feces has also been shown [Okamoto et al., 1998a]. Taken together, TTV may be transmitted not only parenterally but also nonparenterally. Further studies of maternal and sexual contact and fecal-oral transmission are needed to establish the exact mode of TTV transmission.

The pathogenic role of TTV is now in the spotlight. The original study showed the ability of TTV to cause post-transfusion hepatitis based on the association of TTV viremia and aminotransferase increases [Nishizawa et al., 1997]. Although our investigation documented frequent TTV coinfection in HCV carriers undergoing hemodialysis, none had clinically apparent symptoms associated with TTV infection. No difference was observed in aminotransferase levels between TTV-positive and -negative patients, and the levels were within the corrected normal range in approximately 70% of each group. The follow-up study further demonstrated the lack of association of ongoing TTV infection with disease. No apparent increase of aminotransferase levels was observed after TTV infection. Resolution of aminotransferase elevation was not evident in patients who became clear of TTV. The findings suggest that asymptomatic TTV carriage can occur frequently in HCV carriers undergoing hemodialysis and that coinfection with TTV does not enhance virulence. It has been suggested that TTV may replicate in hepatocytes, because its DNA was detected in the liver in titers from 10 to 100 times higher than the corresponding serum [Okamoto et al., 1998b]. Intrahepatic replication of TTV may affect that of HCV in coinfected patients. In this study, however, the levels of HCV replication were not affected by the presence of ongoing TTV coinfection, thus indicating the lack of an interference phenomenon. The exact pathogenic role of TTV as a hepatitis-associated virus and its replication in the liver is still doubtful and awaits further study for clarification.

The original study suggested a propensity for TTV to establish only transient infections in blood recipients because TTV viremia was cleared after 15–17 weeks in two of the three patients investigated for post-transfusion non-A-C hepatitis [Nishizawa et al., 1997]. In another report, however, TTV infection was found in hemophilic patients who were tested 10 years after their last exposure to nonvirally inactivated clotting factor concentrates [Simmonds et al., 1998], indicating that infection may also be persistent in a certain pro-

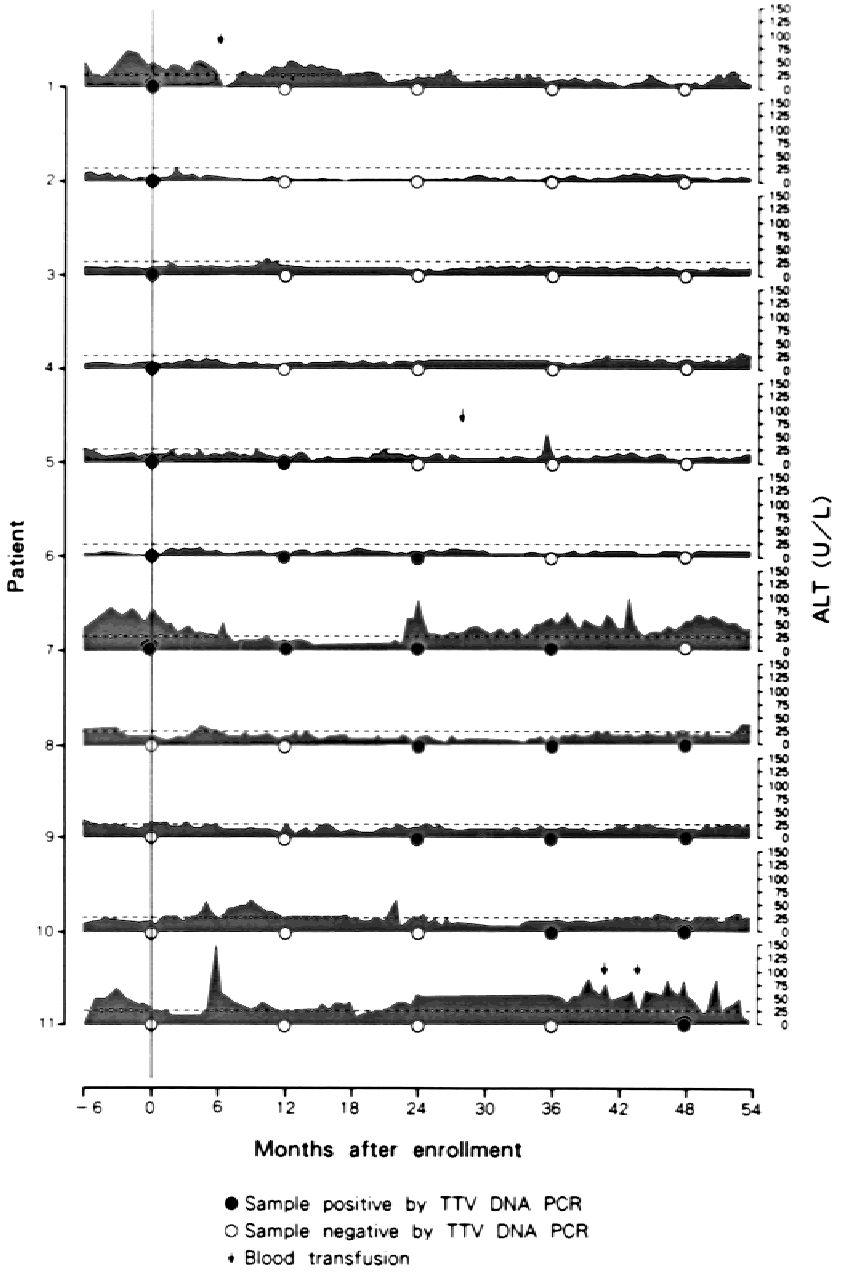

Fig. 2. Pattern of serum alanine aminotransferase (ALT) levels relative to changes in detection of serum transfusion-transmitted virus (TTV) DNA during follow-up. Persistent hepatitis C virus infection was observed in each patient. The dashed lines indicate the corrected upper normal limit for ALT.

portion. Thus, the natural course of TTV infection is largely unresolved. In the present study, TTV DNA sequences in serum samples were cleared in 29% (7/24) of the viremic patients over the 4-year follow-up period, suggesting an annualized rate of TTV clearance of approximately 7.3% (95% CI 0.8–25.5%). The probability of TTV clearance was higher than that of HCV clearance. In contrast with HCV infection, TTV viremia seems to disappear with time for a considerable proportion of patients. Nonetheless, the data obtained showed the propensity of TTV for establishing long-term infection in hemodialysis patients, which may account, together with the occurrence of infection, for the relatively high prevalence of infection in this population. Although the data obtained suggest possible occurrence of TTV clearance as well as new infection in the study population, there is a possibility that some of the losses of viremia and the apparently new infections may be due to natural fluctuation in viral load and a low level of TTV replication may persist with viral expression only in hepatocytes or other cells. More detailed analysis of TTV viremic levels and the development of antibody tests to show past infection and immunity should shed further light on the natural history

of TTV infection. Further studies on stability of TTV variants, which may affect PCR results, are also necessary to address this issue.

TTV infection, like that of hepatitis G, has been shown to be common worldwide but its association with disease is not clear. Strong evidence of asymptomatic carriage and spontaneous resolution suggests that TTV infection is harmless clinically and that there are still more human hepatitis-associated viruses to be found. The findings, however, do not rule out the possibility of more aggressive liver disease in susceptible individuals. Genetic variants of TTV [Okamoto et al., 1998b] may also vary in their ability to cause disease. Again, more analysis of genetic variants of TTV and the preparation of TTV antigens that can be the basis of antibody tests are together likely to provide valuable information about the nature and prevalence of TTV infection.

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